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Harnessing the catabolic diversity of rhodococci for environmental and biotechnological applications

Robert van der Geize and Lubbert Dijkhuizen*

The field of *Rhodococcus* cell engineering is rapidly advancing because of the availability of improved genetic tools and increased insights in their broad catabolic and biochemical diversity. Rhodococci harbor large linear plasmids that may contribute to their catabolic diversity. In addition, multiple pathways and gene homologs are often present, thus further increasing *Rhodococcus* catabolic versatility and efficiency. The recent development of effective genetic tools for *Rhodococcus*, such as unmarked gene deletion, transposon-based mutagenesis, and gene expression systems, now allows the construction of biocatalysts with desirable properties for industrial purposes. This is exemplified here by a description of cell engineering of biocatalysts for improved desulphurization and steroid biotransformation.

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Abbreviations

9OHAD	9α-hydroxy-4-androstene-3,17-dione
AD	4-androstene-3,17-dione
ADD	1,4-androstadiene-3,17-dione
BT	benzothiophene
DBT	dibenzothiopene
DHBD	2,3-dihydroxybiphenyl 1,2-dioxygenases
NTH	naphtho[2,1-*b*]thiophene
ORF	open reading frame
PCB	polychlorinated biphenyls

Introduction

Members of the genus *Rhodococcus* occur widely, and are aerobic, non-sporulating bacteria, with a high G+C content. Rhodococci are of environmental and biotechnological importance because of their broad catabolic diversity and array of unique enzymatic capabilities [1,2]. Many applications are found in the environmental, pharmaceutical, chemical and energy sectors. Rhodococci are well-suited industrial biocatalysts because of their robustness and their exceptional ability to degrade hydrophobic natural compounds and xenobiotics, including polychlorinated biphenyls (PCBs). Rhodococci are well-established industrial organisms for the large-scale production of acrylamide and acrylic acid. They also are good candidates for the industrial production of bioactive steroid compounds [3]. Over the years, advances in *Rhodococcus* genetics were relatively slow [4,5], but effective tools have become available recently.

In this review, we discuss the apparent redundancy in catabolic pathways and genes observed in rhodococci, giving them their broad metabolic diversity and the important role that large linear plasmids may play herein. The application of effective genetic tools for rhodococci, such as unmarked gene deletion and transposome-complex based methods is described, that have enabled *Rhodococcus* researchers to successfully engineer useful biocatalysts for desulphurization and steroid biotransformation.

Metabolic diversity of rhodococci is related to the presence and mobilization of large linear plasmids

Recent whole genome sequence analysis of *Rhodococcus* sp. strain RHA1 (9.7 Mb) (http://www.bcgsc.bc.ca/cgi-bin/rhodococcus/blast_rha1.pl), *Rhodococcus aetherovorans* strain I24 (7 Mb) (J Archer, personal communication), *Rhodococcus erythropolis* strain PR4 (7 Mb) (S Harayama, personal communication), and additional experimental data in the literature, have shown that *Rhodococcus* strains harbor a variety of large, mostly linear plasmids. The most effective PCB degrader, *Rhodococcus* sp. strain RHA1, contains three linear plasmids pRHL1 (1100 kb), pRHL2 (450 kb) and pRHL3 (330 kb) harboring biphenyl/PCB degradative *bph* genes, many of which encode dioxygenase enzymes. The *bph* genes are scattered throughout the RHA1 genome and are located on the chromosome as well as on linear plasmids pRHL1 and pRHL2 [6]. Genes encoding isopropylbenzene degradation (*ipb*) and an *etbD1* homolog, involved in biphenyl degradation, were identified on a large linear plasmid pBD2 (210 kb) of *R. erythropolis* strain BD2 [7••]. The complete nucleotide sequence of pBD2 revealed a total of 212 open reading frames (ORFs), with putative catabolic functions for 23 ORFs and an even greater number of ORFs (32) with putative functions in transposition events. Functional analysis of pRHL2 suggests that linear plasmids may well function as a determinant of propagation of the diverse degradative genes among the rhodococci [6]. Moreover, the similarities found in the key enzymes and in the

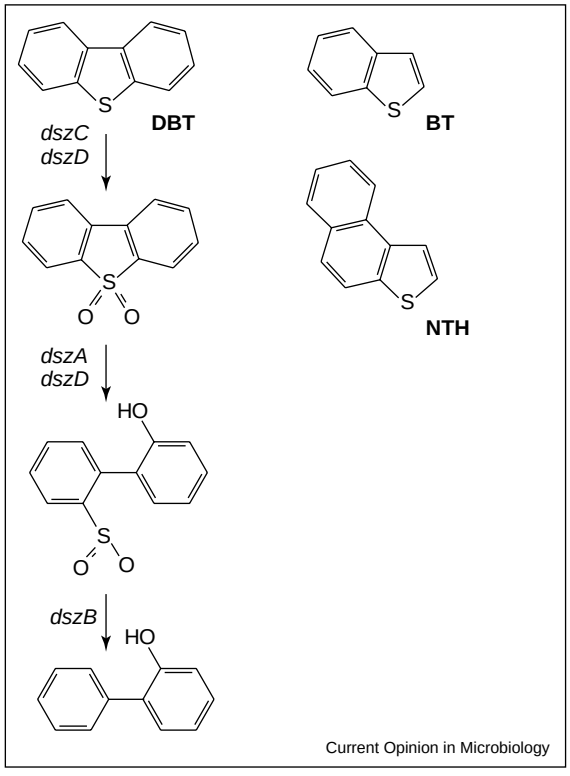
regulators of the isopropylbenzene catabolic pathway genes in *R. erythropolis* BD2 and the linear plasmid encoded functions of biphenyl degradation pathways, indicate that the *ipb* and *bph* operons have been distributed among Gram-positive soil bacteria via linear plasmid mediated horizontal gene transfer [7••]. More examples of plasmid-borne catabolic pathways in *Rhodococcus* have been reported. *Rhodococcus* sp. strain IGTS8, for example, harbours a large 150 kb plasmid that is involved in the desulphurization of organosulphur compounds [8]. *Rhodococcus* sp. strain DK17 harbors two large plasmids, pDK1 (380 kb) and pDK2 (330 kb), the latter carrying genes encoding the initial oxygenase and meta-ring cleavage dioxygenase steps in alkylbenzene metabolism [9]. The wide catabolic diversity of *Rhodococcus* species therefore partly owes to the presence and mobility of these large linear plasmids. It should be noted however that this diversity does not solely relate to large linear plasmids. Other features contribute to the considerable gene diversity of rhodococci. Considerable redundancy is observed in the genome sequences noted, with multiple copies of many genes on plasmids and the chromosome (e.g. many copies of TCA cycle enzymes in central metabolism).

Multiple homologs of enzymes in catabolic pathways further enhance *Rhodococcus* versatility

Rhodococcus genomes encode large numbers of oxygenase enzymes, many of which may be functional homologs. The presence of four alkane monooxygenase genes (*alkB1–alkB4*) has been reported for *Rhodococcus* sp. strain Q15 and *R. erythropolis* strain NRRL B-16531, encoding similar, but not identical, enzymes of similar size displaying high amino acid sequence homology [10••]. Three to five alkane hydroxylase homologs have been identified in eight other *Rhodococcus* strains. Therefore the presence of multiple alkane hydroxylases may well be a common feature of *Rhodococcus* strains [11•]. The number of *alkB* homologs present appears to correlate with the metabolic diversity of the strain (i.e. the range of *n*-alkanes that can be metabolized). *R. erythropolis* strain SQ1 and *R. rhodochrous* strain DSM43269 both degrade steroids and were found to contain three and four 3-ketosteroid 9α-monooxygenases, respectively, sharing 50–60% amino acid sequence identity (Van der Geize *et al.*, unpublished). Three 2,3-dihydroxybiphenyl 1,2-dioxygenases (DHBD) were characterized from the PCB degrading *R. globerulus* strain P6, encoded by the *bphC1* (DHBD-I), *bphC2* (DHBD-II) and *bphC3* (DHBD-III) genes. Recent studies indicated that the presence of multiple DHBD isoenzymes in *R. globerulus* strain P6 improved its PCB-degrading capabilities [12,13]. In *R. erythropolis* strain YK2 five extradiol dioxygenase genes (*edi1*, *edi2*, *edi3*, *edi4* and *dfdB*) have been identified, with some of the gene products displaying similarities to DHBD [14]. Besides many biphenyl dioxygenases, *Rhodococcus* sp. strain RHA1 harbors two nearly identical 2-hydroxy-6oxohepta-2,4-dienoate hydrolase genes (*etbD1*, *etbD2*) [15]. In addition to the chromosomally located *bphGF1E1* gene cluster, a second set of *bphE2F2* genes was identified downstream of *bphD1* in strain RHA1. The first set encodes the primary 2-hydroxypenta-2,4-dienoate metabolic pathway of biphenyl and ethylbenzene degradation, whereas the *bphE2F2* genes are probably not essential for biphenyl degradation [16,17]. Strain YK2 was shown to contain three hydrolase-like genes, two of which were clustered with extradiol dioxygenase genes [14].

In contrast to the highly homologous biphenyl degradation genes (*bph*) in the clusters of *R. globerulus* P6, *Rhodococcus* sp. strain RHA1 and *R. erythropolis* TA421, the mapping order and sequences of the *bph* genes in *Rhodococcus rhodochrous* strain K37 are clearly different. This was taken to suggest that this *R. rhodochrous bph* gene cluster evolved separately from the well-known *bph* gene clusters of the other three strains [18]. *Rhodococcus opacus* strain 1CP contains a cluster of four chlorocatechol catabolic genes that are only distantly related to the known *Rhodococcus* genes encoding chlorocatechol enzymes. They appear to represent a new evolutionary line of 3-chlorocatechol catabolic enzymes [19].

Figure 1

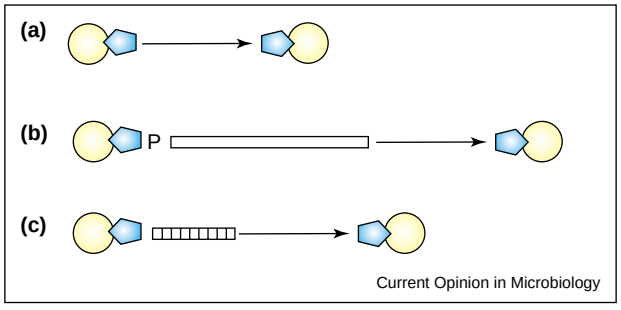


Proposed general degradation pathway of DBT by *Rhodococcus*, adapted from [26]. Chemical structures of BT and NTH are shown in the panel on the right.

Optimizing biodesulphurization by rhodococci for a better environment

There is considerable interest in developing a biocatalytic system as precombustion technology for the specific removal of organic sulphur from coal and petroleum products. Sulphur oxides generated by combustion of fossil fuel contribute to acid rain and air pollution. Hydrodesulphurization of fossil fuels results in the formation of the recalcitrant cyclic compounds benzothiophene (BT), dibenzothiopene (DBT) and 4,6-dimethyldibenzothiophene. *Rhodococcus* strains are metabolically diverse with respect to their desulphurization capabilities. The substrate specificities of enzymes involved in desulphurization of BT, DBT and their derivatives, were suggested to be different in *Rhodococcus* sp. strain KT462 and *R. erythropolis* KA2-5-1. *Rhodococcus* sp. strain KT462 can use both BT and alkylated forms of BT as a sole source of sulphur, whereas *R. erythropolis* KA2-5-1 is unable to degrade BT, but can desulphurize alkylated forms of (D)BT [20,21]. *Rhodococcus* sp. strain WU-K2R and *Rhodococcus* sp. strain T09 also differ clearly in desulphurization, despite the fact that 16S ribosomal DNA of strain T09 is 99.9% identical to that of strain WU-K2R [22]. Strain WU-K2R desulphurizes BT and an asymmetric structural isomer of DBT, naphtho[2,1-*b*]thiophene (NTH), whereas *Rhodococcus* sp. strain T09 desulphurizes BT, but not NTH.

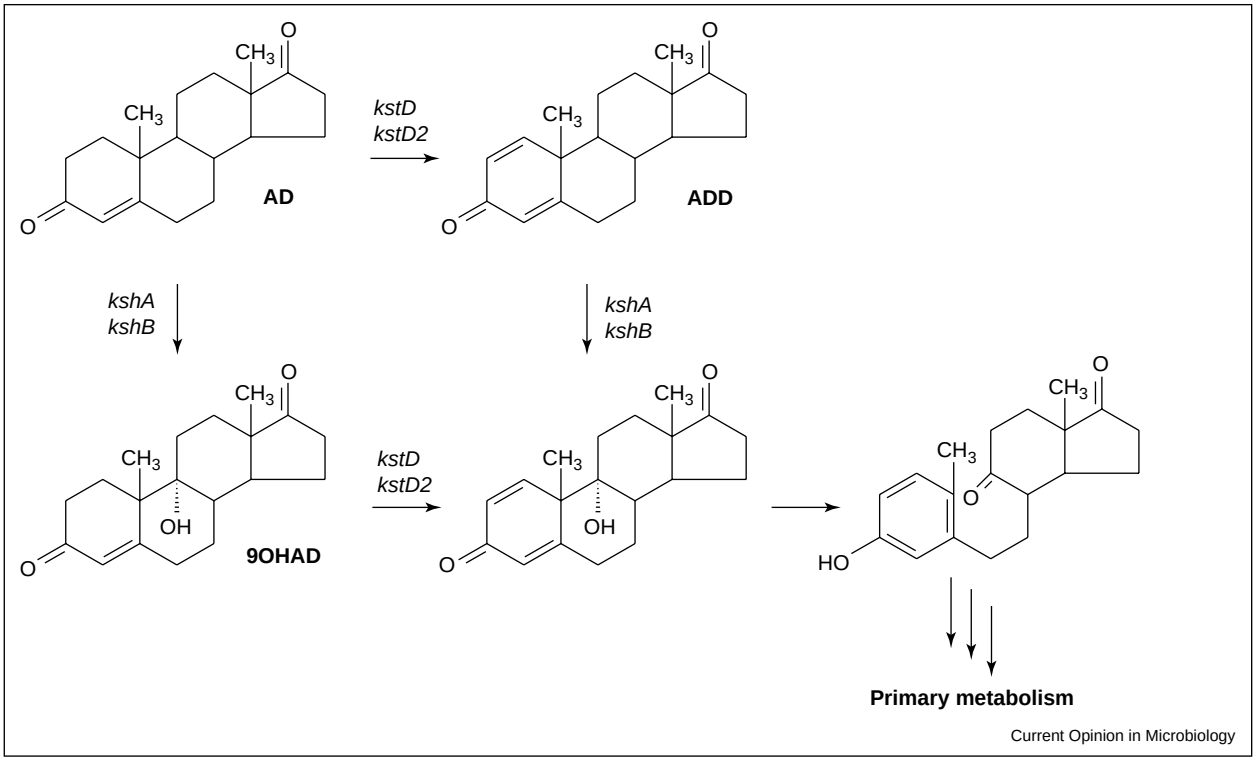
Figure 2



Schematic drawing of the transposome complex method used in transposon mutagenesis [30••]. **(a)** gene expression [28•], **(b)** or as a promoter-probe system [29••] **(c)** in *Rhodococcus*. The transposome is mobilized to *Rhodococcus* by electrotransformation and stably integrated into the genome upon transposition. Closed circle, transposase enzyme; pentagon, transposon outer end; open bar, single gene or gene cluster; striped bar, promoter-less reporter gene; P, promoter; black arrow, resistance marker. Adapted from [28•].

In recent years, genetically engineered DBT desulphurizing rhodococci have been constructed, aiming to enhance desulphurization. The genes encoding DBT desulphurization have been named *sox* (sulphur oxidation) [23] or

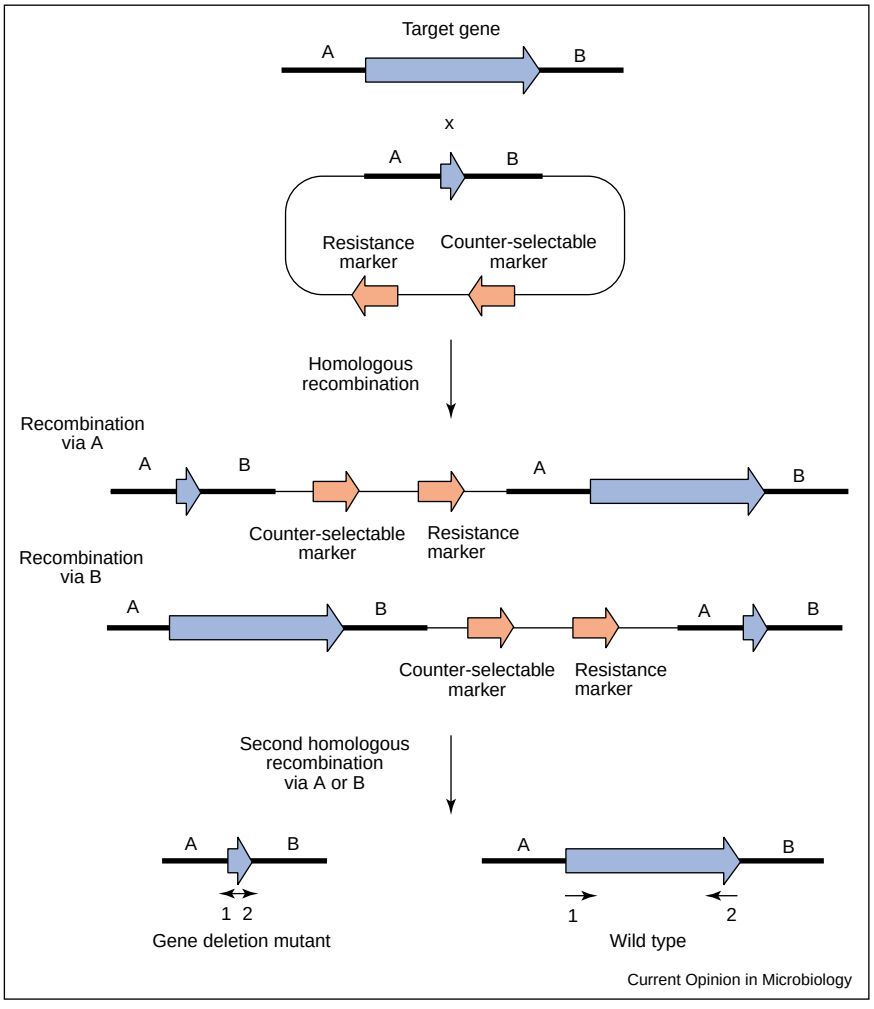
Figure 3



Proposed degradation pathway of the steroid compound 4-androstene-3,17-dione (AD) by *Rhodococcus erythropolis* strain SQ1. *kstD* and *kstD2* encode two 3-ketosteroid Δ^1-dehydrogenase isoenzymes. *kshA* and *kshB* encode the two-component enzyme system 3-ketosteroid 9α-monooxygenase. Adapted from [33••].

dsz (desulphurization) (Figure 1) [24] and are plasmid-borne in *Rhodococcus* sp. strain IGTS8 (150 kb plasmid) and other *Rhodococcus* strains (100 kb plasmids) [8]. Various sulfur compounds, such as inorganic sulfate, methionine and cysteine, repressed desulphurization [25]. Strains with de-regulated and/or enhanced expression of the *dsz* genes were therefore needed. The *dszABC* gene cluster, encoding a monooxygenase, a desulphinase and another monooxygenase, respectively, and the related *dszD* gene, encoding a flavin reductase, from *R. erythropolis* strain KA2-5-1 have been re-introduced into strain KA2-5-1 on a pRC4 (*R. rhodochrous* strain IFO3338 derived) shuttle vector and efficiently expressed. The resulting recombinant strain, containing two copies of the *dszABC* gene cluster and one copy of the *dszD* gene, showed a four-fold higher DBT desulphurization ability than strain KA2-5-1 [26]. Matsui *et al.* [27•] constructed a recombinant strain of *Rhodococcus* sp. strain T09 expressing the *dszABC* and *dszD* genes on *Rhodococcus–E. coli* shuttle vector pRHK1 [26]. The *rrn* promoter region of the 16S ribosomal RNA gene was used to drive *dszABCD* gene expression, enabling the recombinant strain to desulphurize DBT even in the presence of methionine, cysteine or inorganic sulphate as a source of sulphur. Similarly, Watanabe *et al.* [28•] expressed the *dsz* gene cluster of KA2-5-1 under control of the *kapI* promoter, which is not repressed by sulphate. The *kapI* promoter of strain KA2-5-1 was isolated via a transposon-based promoter-probe system using red-shifted *gfp* as a reporter gene (Figure 2c). The P_{kapI}-*dszABCD* expression cassette

Figure 4



Scheme detailing the unmarked gene deletion method for *Rhodococcus* by the double recombination strategy using a counter-selectable marker (e.g. *sacB*) [34]. A resistance marker is used to select for the first homologous recombination event, which may occur at either side of the targeted gene (i.e. via A or B). The resulting recombinant is subsequently grown overnight, under non-selective pressure, to allow the rare second homologous recombination event (either via A or B) to occur, resulting in wild type or the gene deletion mutant phenotype. Subsequent plating on counter-selective medium (i.e. with sucrose) will allow growth of mostly double crossover recombinants, although some recombinants may arise from an inactivated counter-selection marker. Colony PCR using primers 1 and 2 can be easily performed to select for the unmarked gene deletion mutant.

was transferred to *R. erythropolis* strain MC1109 using either a transposome-based method (Figure 2b) or shuttle vector pRHK1 [28•,29••]. Recombinant strains from both methods showed an approximate two-fold increase in DBT desulphurization activity compared to parent strain KA2-5-1. The transposome method also proved useful in isolating random mutants (Figure 2a) of *R. erythropolis* strain KA2-5-1 [30••] and *Rhodococcus equi* [31]. The strain KA2-5-1 mutants, expressing the desulphurization phenotype in the presence of sulphate, were shown to have a disrupted *cbs* gene, encoding cystathionine β-synthase, which is part of the trans-sulphurization pathway converting homocysteine into cysteine. It is now believed that only cysteine and sulphite contribute to repression, and that *cbs* inactivation results in a reduction of the amount of cysteine in cells, resulting in desulphurization derepressing [30••].

Cell engineering of *Rhodococcus* biocatalysts by inactivating multiple (iso)enzymes by gene deletion

Besides enhancing and (de)regulating the expression of catabolic pathway genes, specific inactivation of undesirable enzyme activity steps is also generally important for the construction of strains suitable for industrial production processes for high-value pathway intermediates. As outlined above, rhodococcal catabolic pathways are of high complexity and may contain isoenzymes. This necessitates the sequential inactivation of multiple genes. Bacterial strains performing sterol-steroid transformations, for example, need to be blocked at the level of steroid polyaromatic ring structure opening. Otherwise, catabolic activities in the strain will cause a substantial, if not complete loss of substrate and desired product. Enzymatic steps in steroid ring degradation by *R. erythropolis* strain SQ1 involve two 3-ketosteroid Δ^1-dehydrogenase isoenzymes, encoded by *kstD* and *kstD2*, and a two-component 3-ketosteroid-9α-hydroxylase, encoded by *kshA* and *kshB* (Figure 3) [32,33••]. An unmarked gene deletion method, using *sacB* as a positive selection marker, was developed for *Rhodococcus*, enabling the isolation of mutants blocked in multiple steps (Figure 4) [34]. Intergeneric conjugation between *E. coli* S17-1 and *Rhodococcus* species was suggested to be of crucial importance to minimize random integration of the construct used [34,35]. Single *kstD* or *kstD2* gene deletion mutants showed that the presence of either gene can promote degradation and growth on the steroid compounds 4-androstene-3,17-dione (AD) and 9α-hydroxy-4-androstene-3,17-dione (9OHAD) [32,36]. Deletion of both genes, however, completely inhibited growth on these steroid substrates. AD biotransformation by the *kstD kstD2* double mutant resulted in sustained 9OHAD accumulation in high (>90%) yields [32]. Gene deletion of either *kshA* or *kshB* in *R. erythropolis* SQ1 was shown to completely inhibit growth on AD as well as on 1,4-androstadiene-3,17-dione (ADD), while growth on 9OHAD was not blocked [33••]. Accumulation of ADD (30–50%) was observed in AD biotransformation experiments with *kshA* and *kshB* mutant strains. A *kshA kstD kstD2* triple gene deletion mutant strain was additionally constructed that was fully blocked in steroid polyaromatic ring degradation.

The same gene deletion technology has also been applied in *R. rhodochrous* to inactivate multiple gene homologs involved in steroid degradation (van der Geize *et al.*, unpublished) and in *R. opacus* strain HL PM-1 to delete the transcriptional regulatory gene *npdR* involved in picric acid degradation [37,38•].

Conclusions

Members of the genus *Rhodococcus* are well known for their extensive catabolic diversity, and as very promising robust biocatalysts for industrial chemical production. Extensive information is available in the literature about the presence of multiple homologous pathways and various isoenzymes in rhodococci, often located on plasmids. Evidence is available that suggests these plasmids may also contribute to propagation and mobilization of genes encoding these catabolic pathways and enzymes between rhodococci. In an exciting development, the first complete *Rhodococcus* genome sequences are just coming available, revealing very large genome sizes, partly owing to the presence of (multiple) large (linear) plasmids. Further analysis of these genome sequences will undoubtedly improve insights in the basis of this catabolic complexity and diversity, and its genomic organisation. This will greatly support attempts to construct *Rhodococcus* strains with suitable properties for environmental and biotechnological applications. With the recent emergence of effective gene technology for various rhodococci, rational cell engineering is becoming increasingly feasible. This will allow harnessing of the catabolic diversity of rhodococci, involving overexpression of key catabolic pathways and enzymes, as well as inactivation of undesirable pathways/enzymes, resulting in optimalization of biocatalyst properties.

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This paper reports the complete nucleotide sequence of a rhodococcal linear plasmid revealing a large number of open reading frames (ORFs) involved in transposition and catabolism. The authors suggest that certain catabolic pathways may have been distributed among Gram-positive soil bacteria via linear plasmid-mediated horizontal gene transfer. The presence of a large number of transposon-related ORFs on this linear plasmid suggests that such plasmids can undergo dynamic rearrangements at a high frequency.

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This study presents a strong example of the apparent redundancy in catabolic genes present in the genus *Rhodococcus*. At least four alkane hydroxylases *alkB* gene homologs were found in two rhodococcal strains. Characterisation of these alkane hydroxylases suggests that the presence of several homologs broadens the catabolic capabilities of the strain. Several gene homologs were identified in eight other *Rhodococcus* strains, indicating that multiple alkane hydroxylases may be a common feature of *Rhodococcus* strains.

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